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从天然提取物或分离部位中以烯醇式丙酮酸转移酶为靶点的抗细菌活性筛选*

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摘要：目的是以烯醇式丙酮酸转移酶（EPT）为靶点筛选其抑制剂，以期寻找抗细菌活性样品。实验是在96孔酶标板上对来源于169个科、560个属、916种动植物2490个提取物或分离部位样品在EPT模型上进行了批量筛选。结果表明在96.15 μg/ml浓度下发现了来缘于80个科、169个属、218个种的309个样品有活性，其中14个样品的IC_{50}小于10.00 μg/ml，40个样品的IC_{50}在10.01～30.00 μg/ml范围，83个样品的IC_{50}在30.01～50.00 μg/ml范围，172个样品的IC_{50}在50.01～96.15 μg/ml范围。通过以上工作我们认为以烯醇式丙酮酸转移酶为分子靶点的体外筛选方法稳定、方便、快速、微量、有效，特别适用于天然产物的抗细菌活性筛选。

关键词：抗细菌活性筛选；烯醇式丙酮酸转移酶；天然产物；提取物；分离部位
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Searching for Antibacterial Activities of Extracts and Fractions Derived from Natural Sources Targeting Enoylpyruvate Transferase*

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Abstract：To discover inhibitors of enoylpyruvate transferase with antibacterial activity a batch of 2490 extract or fraction samples prepared from plants and animals belonging to 169 families，560 genera and 916 species were tested on enoylpyruvate transferase bioassay in 96-well microtiterplates. Finally 309 samples，

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which belong to 80 families, 169 genera and 218 species, showed inhibitory activity at 96.15 μg/ml, in which 14 samples showed IC_{50} at = < 10.00 μg/ml, 40 samples showed IC_{50} at 10.01 – 30.00 μg/ml, 83 samples showed IC_{50} at 30.01 – 50.00 μg/ml and 172 samples showed IC_{50} at 50.01 – 96.15 μg/ml. It is indicated that this in-vitro bioassay is convenient, stable, rapid, sensitive and effective in searching for antibacterial activity samples from natural sources.

Key words: Antibacterial activity screening; Enoylpyruvate transferase; Natural products; Extracts; Fractions

Various agar solid media screening methods are the common means in cellular level to look for antibacterial activity. Recently, molecular target assays are emerging and used for antibacterial activity screening including targets such as N-acetyl-glucosamine-1-phosphate uridyl transferase (Sulzenbacher *et al*, 2001), uridine 5'-diphospho-N-acetyl-enolpyruvyl glucosamine reductase (Benson *et al*, 1995), uridine 5'-diphospho-3-O-[R-3-hydroxymyristoyl]-N-acetyl-glucosamine deacetylase (Chen *et al*, 1999), peptide deformylase (Yuan *et al*, 2001), and UMP kinase (Bucurenci *et al*, 1998).

It is well known that bacteria, but not mycoplasma or mammals including humans, have a cell wall. Peptidoglycan is one of the main structural components of the cell wall. Inhibition of peptidoglycan synthesis can influence the formation of cell wall, which can, as a consequence, kills bacteria. Therefore, screening based on main enzymes involved in peptidoglycan synthesis is an attractive approach to discover new antibacterial agents (Chandrakala *et al*, 2001) with no harm to humans. Enoylpyruvate transferase (EPT) is one of the key enzymes acting in the first stage of peptidoglycan synthesis of the cell wall. It is a single polypeptide with a molecular weight of 41, 000Da (Zemell *et al*, 1975).

In this paper we describe testing of natural extract or corresponding fraction samples with the EPT bioassay in order to discover new antibacterial activity samples from natural products.

1 Materials and Methods

Materials and Instruments *Enterobacter cloacae* enoylpyruvate transferase (EPT) was provided by BAYER AG. Uridine 5'-diphospho-N-actetylglucosamine (UDPAG) was purchased from SIGMA (No. U-4375) and phosphoenolypyruvic acid monopotassium salt (PEP-K) from FLUKA (No. 79415). Other reagents and solvents used in the experiments are of biological, analytic and reagent grades.

2490 samples tested are extracts or fractions prepared from plants and animals belonging to 169 families, 560 genera and 916 species. They are a part of the sample library of the Lab. for Screening within the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, China.

SPECTRAmax 340 96-well microtiterplate reader from Molecular Devices (USA) was used for end point measurement.

Sample preparation 20 mg sample of extracts or fractions was dissolved in 2 ml of Me_2SO as sample stock solution (10 mg/ml). The final concentration of sample for pre-test was 96.15 μg/ml, in which 2 μl sample solution (diluted to 2.5 mg/ml by adding Me_2SO) was added to microtiterplate wells as appropriate.

EPT bioassay The assay employed is a microtiterplate adaptation of a phosphate detection method described previously (Lanzetta *et al*, 1979).

Two μl Me_2SO solvent were distributed in Blank wells (Bl) and Substrate wells (Sub). Two μl sample were filled in Sample wells (Sam) and Sample Blank wells (Samb). Fifty μl buffer mixture, which contains 25 μl of 50 mmol/L Tris (pH7.4) and 25 μl of 20% BSA-Tris, were added to Blank wells and Sample Blank wells. Fifty μl bioassay mixture, which contains 12.5 μl of 1 m mol/L UDPAG, 12.5 μl 260 μmol/L PEP-K and 25 μl of 4 μg/ml EPT, were added to Substrate wells and Sample wells. After incubation at 37℃ for 2 h, 100 μl indicator containing 0.045% Malachite Green Base (MGB) and 3.16% Ammonium Molybdate Tetrahydrate (AMT) was added to each well of a 96-well microtiterplate, and OD values at 630 nm were measured by a microtiterplate reader.

Sample testing was divided into three steps: 1) Pre-test: Samples were screened in one well at the concentration of 96.15 μg/ml. Samples with ≥ 40% inhibition at 96.15 μg/ml were selected for Follow-up test; 2) Follow-up test: Samples were screened in duplicates at a concentration of 96.15 μg/ml. Samples with ≥ 50% inhibition at 96.15 μg/ml were selected for Evaluation-test; 3) Evaluation-test: Samples were screened in triplicates at five concentrations of 96.15, 48.08, 24.04, 12.02 and 6.01 μg/ml. IC_{50} (μg/ml) of active samples were calculated using the following formula:

$$IC_{50} = \frac{Concentration_L \ (I_H - 50) + Concentration_H \ (50 - I_L)}{I_H - I_L}$$

Templates of Pre-test, Follow-up test and Evaluation-test are presented in Figs. 1, 2 and 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	Samb1	Samb2	Samb3	Samb4	Samb5	Samb6	Samb7	Samb8	Samb9	Samb10	Samb11
B	B1	Sam1	Sam2	Sam3	Sam4	Sam5	Sam6	Sam7	Sam8	Sam9	Sam10	Sam11
C	B1	Samb12	Samb13	Samb14	Samb15	Samb16	Samb17	Samb18	Samb19	Samb20	Samb21	Samb22
D	B1	Sam12	Sam13	Sam14	Sam15	Sam16	Sam17	Sam18	Sam19	Sam20	Sam21	Sam22
E	Sub	Samb23	Samb24	Samb25	Samb26	Samb27	Samb28	Samb29	Samb30	Samb31	Samb32	Samb33
F	Sub	Sam23	Sam24	Sam25	Sam26	Sam27	Sam28	Sam29	Sam30	Sam31	Sam32	Sam33
G	Sub	Samb34	Samb35	Samb36	Samb37	Samb38	Samb39	Samb40	Samb41	Samb42	Samb43	Samb44
H	Sub	Sam34	Sam35	Sam36	Sam37	Sam38	Sam39	Sam40	Sam41	Sam42	Sam43	Sam44

Fig. 1 Template for Pre-test.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	B1	B1	Samb7	Sam7	Sam7	Samb15	Sam15	Sam15	Samb23	Sam23	Sam23
B	Sub	Sub	Sub	Samb8	Sam8	Sam8	Samb16	Sam16	Sam16	Samb24	Sam24	Sam24
C	Samb1	Sam1	Sam1	Samb9	Sam9	Sam9	Samb17	Sam17	Sam17	Samb25	Sam25	Sam25
D	Samb2	Sam2	Sam2	Samb10	Sam10	Sam10	Samb18	Sam18	Sam18	Samb26	Sam26	Sam26
E	Samb3	Sam3	Sam3	Samb11	Sam11	Sam11	Samb19	Sam19	Sam19	Samb27	Sam27	Sam27
F	Samb4	Sam4	Sam4	Samb12	Sam12	Sam12	Samb20	Sam20	Sam20	Samb28	Sam28	Sam28
G	Samb5	Sam5	Sam5	Samb13	Sam13	Sam13	Samb21	Sam21	Sam21	Sub	Sub	Sub
H	Samb6	Sam6	Sam6	Samb14	Sam14	Sam14	Samb22	Sam22	Sam22	B1	B1	B1

Fig. 2 Template for Follow-test.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	$Samb_{11}$	$Samb_{12}$	$Samb_{13}$	$Samb_{14}$	$Samb_{15}$	$Samb_{31}$	$Samb_{32}$	$Samb_{33}$	$Samb_{34}$	$Samb_{35}$	B1
B	B1	Sam_{11}	Sam_{12}	Sam_{13}	Sam_{14}	Sam_{15}	Sam_{31}	Sam_{32}	Sam_{33}	Sam_{34}	Sam_{35}	B1
C	B1	Sam_{11}	Sam_{12}	Sam_{13}	Sam_{14}	Sam_{15}	Sam_{31}	Sam_{32}	Sam_{33}	Sam_{34}	Sam_{35}	B1
D	B1	Sam_{11}	Sam_{12}	Sam_{13}	Sam_{14}	Sam_{15}	Sam_{31}	Sam_{32}	Sam_{33}	Sam_{34}	Sam_{35}	B1
E	Sub	$Samb_{21}$	$Samb_{22}$	$Samb_{23}$	$Samb_{24}$	$Samb_{25}$	$Samb_{41}$	$Samb_{42}$	$Samb_{43}$	$Samb_{44}$	$Samb_{45}$	Sub
F	Sub	Sam_{21}	Sam_{22}	Sam_{23}	Sam_{24}	Sam_{25}	Sam_{41}	Sam_{42}	Sam_{43}	Sam_{44}	Sam_{45}	Sub
G	Sub	Sam_{21}	Sam_{22}	Sam_{23}	Sam_{24}	Sam_{25}	Sam_{41}	Sam_{42}	Sam_{43}	Sam_{44}	Sam_{45}	Sub
H	Sub	Sam_{21}	Sam_{22}	Sam_{23}	Sam_{24}	Sam_{25}	Sam_{41}	Sam_{42}	Sam_{43}	Sam_{44}	Sam_{45}	Sub

Fig. 3　Template for Evaluation-test.

2　Results

Screening of 2490 samples　2490 samples were screened in Pre-test on 57 96-well microtiterplates and 1276 samples were selected for Follow-up test with $\geqslant 40\%$ inhibition. 1276 samples were screened in Follow-up test on 46 96-well microtiterplates and 736 samples were selected for Evaluation-test with $\geqslant 50\%$ inhibition. 736 samples were screened in Evaluation-test on 184 96-well microtiterplates and 309 samples showed inhibitory activities on EPT. Hit rate of active samples is 12.41%. Fourteen samples showed IC_{50} at less than 10.00 μg/ml, 40 samples showed IC_{50} at 10.01 – 30.00 μg/ml, 83 samples showed IC_{50} at 30.01 – 50.00 μg/ml and 172 samples showed IC_{50} at 50.01 – 96.15 μg/ml, respectively.

Activity-sample source relationship　2490 samples of extracts or fractions were prepared from plants and animals belonging to 169 families, 560 genera and 916 species, notably, plants from Annonaceae, Compositae, Euphorbiaceae, Labitae, Liliaceae, Papilionaceae, Ranunculaceae, Rosaceae, Rubiaceae and Umbelliferae. 309 samples, which belong to 80 families, 169 genera and 218 species, showed inhibitory activity at 96.15 μg/ml, notably, samples from Compositae, Euphorbiaceae, Labitae, Polygonaceae, Rosaceae, Rubiaceae and Vitaceae.

3　Discussion

Peptidoglycan is a polymer of a repeating uridine 5 ’-diphospho-N -acetyl glucosamine (UDP-GlcNAc) and uridine 5 ’-diphospho-N-acetyl muramyl pentapeptide (UDP-MurNAc-pp) units cross-linked by short peptide bridges. Enoylpyruvate transferase (EPT) can catalyze the first reaction from UDP-GlcNAc to UDP-MurNAc-pp under phosphoenolypyruvic acid monopotassium salt (PEP-K) as another substrate, liberating $HOPO_3^{2-}$ (Fig.4). EPT activity can be measured by a color change from blue to yellow influenced by $HOPO_3^{2-}$ concentration with indicator MGB/AMT on a microtiterplate reader by end point. OD value decreases if certain samples inhibit EPT reflecting a reduction in $HOPO_3^{2-}$ liberation.

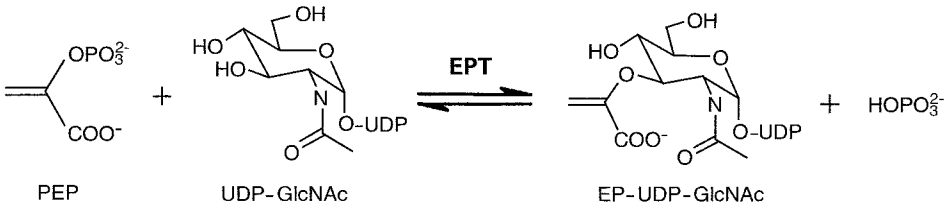

Fig. 4 Mechanism of EPT bioassay.

After screening 2490 samples we found the *in-vitro* bioassay (EPT) described above is a convenient, stable, rapid, sensitive and effective model in searching for antibacterial activity samples from natural sources.

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